

Why do we reject a graft? Role of indirect allorecognition in graft rejection

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CASE PRESENTATION

A 42-year-old Hispanic man had a history of end-stage renal disease secondary to malignant hypertension. He had been on hemodialysis for two years before receiving a 4-antigen mismatched, cadaveric renal allograft four years ago. Positive for cytomegalovirus (CMV), he received a CMV-negative kidney. His serologic hepatitis profile showed positive hepatitis B antibody but negative antigen, and negative hepatitis C antibody. His initial post-transplant course was complicated by delayed graft function and one episode of presumed acute rejection in the first two weeks post transplant, which responded to administration of a steroid pulse.

At discharge after transplantation, his serum creatinine level was at its nadir value of 1.1 mg/dl. Cyclosporine, azathioprine, and steroids were initiated and maintained. He developed post-transplant hypertension, which was managed with an angiotensin-converting-enzyme inhibitor. Approximately four months post transplant, he had another acute rejection episode, which was believed to be secondary to noncompliance with his medications, as reflected by erratic cyclosporine levels during his follow-up clinic visits. Subsequent to that episode, his renal transplant function had been relatively stable (serum creatinine in the 2.1 to 2.6 mg/dl range). But two years ago, progressive

renal transplant dysfunction, as manifested by a slow, progressive rise in serum creatinine, developed; the decreasing renal function was associated with proteinuria (1 to 2 g/24 hr). The erratic cyclosporine levels and blood pressure readings led to the suspicion that he remained noncompliant with his medications.

A renal transplant biopsy one year ago (when his serum creatinine was 4.0 mg/dl and urinary protein-to-creatinine ratio was 1.2) showed evidence of chronic allograft nephropathy (Fig. 1). Specifically, the renal parenchyma was greatly distorted by widespread sclerosis. Most glomeruli were either globally or segmentally obsolescent or appeared hypoperfused. Extensive tubular atrophy was present. The interstitium was expanded by connective tissue and patchy mononuclear cell infiltrates. Isolated tubules showed evidence of tubulitis. Arteries revealed extensive intimal proliferation with near obliteration of the lumens. Small arteries and arterioles revealed concentric layers of connective tissue or prominent hyaline deposition in the wall. Several vessels showed signs of active inflammation of the intima (endotheliitis). Results from the renal biopsy along with the progressive decline in renal function prompted a change in his medications from azathioprine to mycophenolic mofetil (Cellcept), but renal transplant dysfunction progressed, necessitating reinstitution of hemodialysis therapy.

DISCUSSION

DR. MOHAMED H. SAYEGH (*Research Director, Laboratory of Immunogenetics and Transplantation, Renal Division, Brigham and Women's Hospital; and Associate Professor of Medicine, Harvard Medical School, Boston, Massachusetts, USA*): This patient is a typical recipient of a cadaveric renal transplant who ultimately developed chronic allograft nephropathy that led to a second episode of end-stage renal disease. Although chronic allograft nephropathy has been recognized for years, only recently have researchers gone back to the bench to try to understand the pathophysiologic mechanisms of this poorly defined clinicopathologic entity. Chronic allograft nephropathy is clinically characterized by progressive organ dysfunction associated with proteinuria, hypertension, and somewhat typical morphologic changes of graft

Key words: chronic allograft rejection, transplant glomerulopathy, transplant nephropathy, acute rejection.

Presentation of this Forum is made possible by grants from Merck & Company, Incorporated; Astra Pharmaceuticals; Hoechst Marion Roussel, Incorporated; Dialysis Clinic, Incorporated; Amgen, Incorporated; and R & D Laboratories, Incorporated.

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arteriosclerosis, glomerulosclerosis, and variable degrees of interstitial inflammation, fibrosis, and tubular atrophy [1]. Not unique to the kidney, chronic allograft failure affects all solid organ allografts including the heart, lung and, to a lesser degree, the liver [1]. In fact, in other than liver transplant recipients in whom recurrent disease is a major problem, chronic allograft dysfunction is the major cause of graft loss after the first year post transplant. Several studies have established the clinical risk factors of chronic allograft failure. These factors include delayed graft function, acute rejection, infection (such as CMV), and “underimmunosuppression” with acute rejection, particularly recurrent and late episodes (beyond three months) [2–4].

Chronic allograft dysfunction is mediated by both alloantigen-dependent factors (recipient-donor incompatibility, acute rejection, underimmunosuppression) as well as alloantigen-independent factors (ischemia, hypertension, reduced nephron mass, hyperlipidemia, infection, drug nephrotoxicity) [2, 3, 5, 6]. The mechanisms through which alloantigen-independent factors contribute to graft dysfunction have not yet been defined [6]. But it is alloantigen-dependent mechanisms that predominate in initiating and propagating the injury that leads to chronic allograft loss. Perhaps the best evidence supporting a dominant role for alloantigen-dependent factors is the clinical observation that the half-life (defined as the time when 50% of grafts have failed after surviving the first year) of renal allografts decreases with decreasing degrees of HLA matching, that is, incompatibility, in living-related as well as in cadaveric grafts [7]. In addition, patients who demonstrate donor-specific hyporeactivity exhibit a very low incidence of chronic rejection [8]. Experimental evidence from re-transplantation studies in rat models of chronic allograft rejection confirms the importance of alloantigen-dependent mechanisms, particularly in initiating the chronic rejection process [9]. Furthermore, data from several experimental animal models clearly indicate that induction of donor-specific tolerance prevents development of chronic rejection [10]. Therefore, this discussion will focus on alloantigen-dependent mechanisms of allograft dysfunction, particularly the role of indirect allorecognition mechanisms. In that regard it is important to clarify some poorly defined terminology that has been used in the literature. “Chronic allograft dysfunction” is a generic term that does not imply causation. “Chronic rejection,” on the other hand, implies an alloimmune-mediated process driven by the host reaction against graft antigens. “Immunologic” versus “non-immunologic” mechanisms have been used interchangeably with “alloantigen-dependent” versus “-independent” mechanisms, respectively, although this terminology is not entirely accurate, as several alloantigen-independent factors mediate injury through inflammatory mechanisms that involve immune cells and/or their products

[11, 12]. Therefore, the use of the terminology alloantigen-dependent versus alloantigen-independent is preferable. In this Forum, I will be mostly focusing on the role and mechanisms of indirect allorecognition in graft rejection, particularly chronic rejection.

Direct and indirect allorecognition

The primary initial event that ultimately leads to graft rejection is allorecognition (T-cell recognition of alloantigen), in particular, antigens of the major histocompatibility complex (MHC). Several other factors can contribute to the effector mechanisms of graft dysfunction and ultimately failure, however. Using gene knockout animals, Krieger et al showed that CD4⁺ T-cells are essential for initiating allograft rejection [13]. There are two distinct, yet not mutually exclusive, pathways of allorecognition by CD4⁺ T-cells [14–19]. In the so-called “direct” pathway, T-cells recognize intact allo-MHC molecules on the surface of donor antigen-presenting cells (APCs) (Fig. 2). Peptides, derived from endogenous proteins including MHC molecules, bound into the groove of the MHC play an important role in this mode of allorecognition [20]. In the so-called “indirect” pathway, T-cells recognize processed alloantigen presented as peptides by self APCs (Fig. 2). The basic premise for indirect allorecognition as a mechanism for initiation and/or amplification of allograft rejection is that donor alloantigens are shed from the graft, taken up by recipient APCs, and presented to T-cells. The findings in humans [21, 22] and in mice [23] that at least some of the peptides eluted from cell surface class-II MHC molecules represent MHC sequences suggest that processing of MHC molecules is a physiologic event *in vivo*.

Although no evidence has indicated that T-cells recognizing alloantigens via the direct versus indirect pathway are predetermined to be biologically different, from the standpoint of contribution to mechanisms of allograft rejection, these two pathways of allorecognition are distinct for the following reasons: first, differences exist in the microenvironment and locale of “professional” (bone-marrow-derived) APCs (donor versus self) at different times after transplantation; second, direct responses can be primary or primed (secondary) T-cell responses, while indirect responses are all primed (secondary) T-cell responses; third, there can be different effects of immunosuppressive or tolerance regimens on primary versus primed T-cell responses. Therefore, direct and indirect allorecognition need not be mutually exclusive pathways, as each is mediated by different sets of T-cell clones, and both can be involved in the rejection process simultaneously or at different times post transplantation. Mounting evidence indicates that indirect allorecognition, analogous to self-restricted T-cell recognition of nominal antigens, occurs during allograft rejection. I will return to this topic later.

The question is, what are the role and mechanisms of indirect allorecognition in rejection? Early acute allograft rejection might be mediated predominantly by the direct pathway, as the graft contains a significant number of donor-derived passenger APCs (particularly dendritic cells), which express a high density of MHC molecules and can provide the necessary co-stimulatory signals for full T-cell activation [19]. Later, when grafts lack passenger (donor) APCs, T-cells primed by the indirect pathway might play the dominant role in the process of chronic rejection [24]. Definitive evidence proving this hypothesis is lacking, however. Braun et al have demonstrated that “directly” primed CD4⁺ T-cell lines/clones could effect early acute rejection but not chronic rejection of passenger-cell-depleted renal allografts [25]. These studies suggest, but do not prove, an important role for indirect allorecognition in chronic rejection. Other evidence that supports a role for indirect allorecognition in chronic rejection comes from the clinical observation that nominal antigen (for example, viral) recognition, which is analogous to indirect allorecognition, is usually intact in transplant recipients undergoing maintenance immunosuppression, and that *in-vitro* T-cell responses to nominal antigen may correlate with chronic allograft rejection [26–28]. I will address this issue in a moment.

Occurrence of indirect allorecognition

Initial studies in the mouse model showed that adoptive transfer of syngeneic plastic adherent splenocytes primed by the indirect pathway *in vivo* could sensitize a recipient to reject skin allografts in an accelerated fashion [29]. In another report, LEW (RT1^l) rats primed by immunization with soluble class-I or class-II allo-MHC molecules derived from allogeneic DA (RT1.A^{av1}) rats produced antibodies to the soluble allo-MHC molecules and rejected specific skin allografts in an accelerated fashion [30]. Both those studies suggest that self-restricted T-cell recognition of processed allo-MHC molecules plays a role in allograft rejection. The availability of MHC sequences has allowed synthesis of MHC-derived peptides for studying the role and mechanisms of indirect allorecognition in graft rejection. Our initial studies focused on studying the immunogenicity of synthetic class-II MHC allopeptides *in vivo* [31]. Inbred LEW (RT1^l) rats, used as responders, were immunized in the foot pad with a mixture of eight polymorphic synthetic (25mer) class-II MHC allopeptides. These sequences represent the full length of the hypervariable domains of RT1.B^u (DQ and I-A like) and RT1.D^u (DR and I-E like) β chains of the WF (RT1ⁿ) rat. Responder T-cells harvested from popliteal and inguinal lymph nodes of immunized animals exhibited significant proliferation to the MHC allopeptides *in vitro* when presented by self APCs. *In vivo*, peptide-immunized LEW animals mounted significant delayed-type hypersensitivity (DTH)

responses when challenged by the allopeptides, but more interestingly they also had significant DTH responses when challenged by allogeneic WF splenocytes, and not when challenged by syngeneic LEW or third-party allogeneic BN (RT1ⁿ) splenocytes. Follow-up studies on the immunogenicity of these synthetic class-II MHC allopeptides confirm the occurrence of self-MHC-restricted T-cell recognition of processed allo-MHC peptides during vascularized cardiac as well as renal allograft rejection [32, 33]. Splenic CD4⁺ T-cells, taken from LEW recipients of WF vascularized cardiac or renal allografts, proliferate to specific class-II MHC peptides presented by responder APCs. Our studies also demonstrate that not all polymorphic peptides are immunogenic. In fact, in LEW responders, only 2 of the 4 RT1.B^u β and 2 of the 4 RT1.D^u β were immunogenic [31, 33], and immunogenicity of the individual peptides varied in different responder strains. These findings suggested to us that immunogenicity was determined by the responder MHC haplotype [33]. Benichou et al, using mouse class-II MHC allopeptides [34, 35]; Fabre and colleagues [36, 37] and Shirwan et al [38], using rat class-I MHC allopeptides; and Ghobrial et al, using soluble rat class-I MHC molecules [39], have reported similar results indicating the occurrence of indirect allorecognition during rejection of skin and vascularized allografts. More recently, we demonstrated indirect allorecognition in an experimental model of acute rejection due to discontinuation of immunosuppression [40]; this model mimics events in noncompliant patients. In summary, ample evidence indicates that indirect allorecognition occurs during allograft rejection. The question is, what is the role and function of T-cells activated via the indirect pathway in the process of allograft rejection?

Role and mechanisms of indirect allorecognition

Elegant studies by Auchincloss et al, using class-II MHC-deficient mice as donors in a skin allograft model, showed that indirect allorecognition by host CD4⁺ T-cells of donor class-I MHC antigens can initiate rapid skin allograft rejection [41], and that these cells can help generate cytotoxic T-lymphocytes against donor class-I MHC [42]. In a separate study, the same investigators showed that IgG alloantibody production is dependent on CD4⁺ T-cells recognizing peptides of donor antigens through the indirect pathway [43]. Dalloul et al, also using MHC knockout animals, showed that CD4⁺ T-cells can reject skin allografts through indirect allorecognition [44]. Taken together, these data strongly suggest that, in the absence of direct allorecognition, CD4⁺ T-cells primed by the indirect pathway can initiate allograft rejection, and that either class-I or class-II donor antigens can be recognized by CD4⁺ T-cells after processing and presentation by recipient class-II MHC molecules on self APCs.

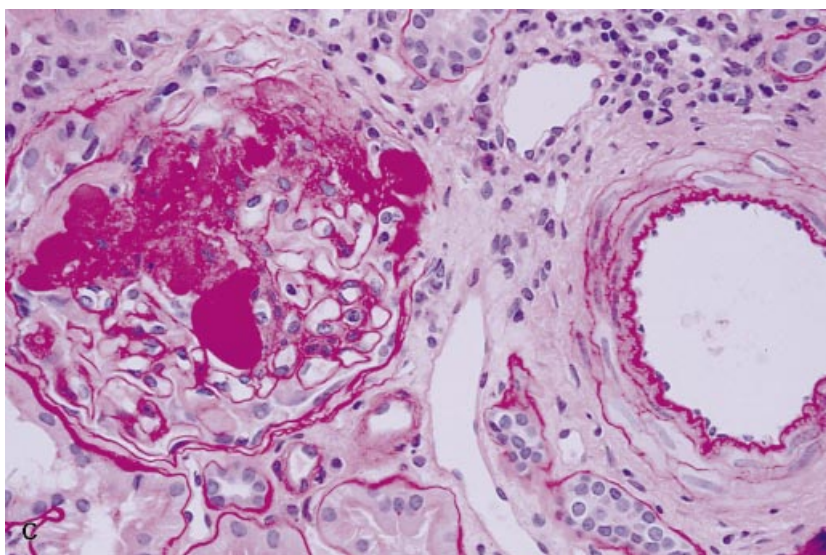
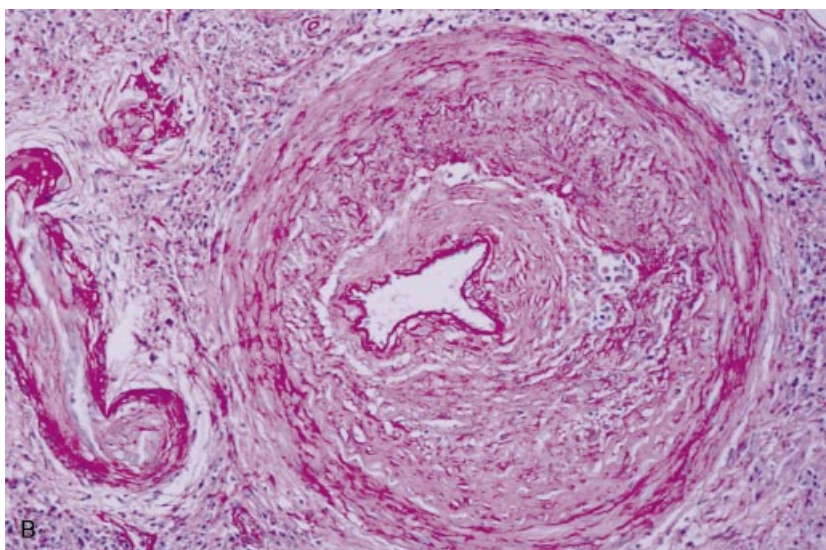
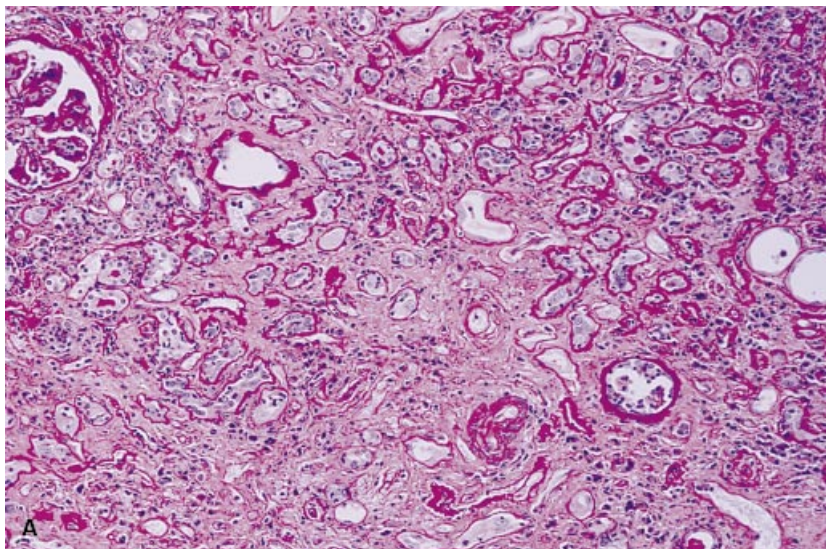


Fig. 1. Graft morphology. (A) This photograph illustrates extensive tubular atrophy. Tubular basement membranes are thickened and the interstitium is expanded by connective tissue. (B) Arteries reveal extensive accumulation of connective tissue in the media and in the expanded intima, resulting in substantial narrowing of the lumen. (C) Glomeruli also reveal obsolescence of the capillary that results in global or segmental glomerulosclerosis. The collapsed capillaries often entrap hyaline material, as depicted in this glomerulus with segmental sclerosis (PAS stain, $\times 400$; courtesy of Dr. H.G. Rennke, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA).

Studies with synthetic class-I MHC peptides derived from the DA rat strain showed that peptide-immunized LEW rats were capable of rejecting DA skin allografts in an accelerated fashion [45]. Furthermore, peptide-immunized recipients rejected renal allografts depleted of donor-derived interstitial dendritic cells [46]. These findings clearly demonstrate that indirect allorecognition contributes to the rejection of vascularized allografts.

Effector mechanisms of transplant rejection involve cellular, delayed-type hypersensitivity (DTH) responses, cell-mediated cytotoxicity, and humoral components [19, 47]. Once fully activated, CD4⁺ T-helper cells produce cytokines that orchestrate various effector arms of the alloimmune response (Fig. 2). Activated CD4⁺ T-cells provide help for CD8⁺ T-cells, B-cells, and monocytes by secreting cytokines and by initiating cell-cell contact-dependent mechanisms. Activated monocytes release a variety of noxious agents that mediate tissue injury. B-cell alloantibody production ultimately results in complement and cell-mediated cytotoxicity. Activated CD8⁺ T-cells kill graft cells in an antigen-specific manner through direct recognition of class-I MHC molecules on target donor cells. Therefore, CD4⁺ T-cells activated via the indirect pathway may effect allograft rejection by DTH and alloantibody-mediated mechanisms independent of direct allorecognition. First, we examined whether priming through the indirect pathway by immunization with donor-derived MHC allopeptides could accelerate acute rejection in an experimental vascularized cardiac allograft model in the rat [48]. We immunized LEW recipients of WF heterotopic cardiac allografts with a mixture of the immunogenic (25mer) WF MHC class-II peptides (RT1.D^ββ1-25, RT1.D^ββ20-44, RT1.B^ββ1-25, and RT1.B^ββ20-44) in complete Freund's adjuvant seven days before transplantation. The control group was immunized with adjuvant and saline or with a mixture of the nonimmunogenic peptides in adjuvant. The animals received cyclosporine, 5 mg daily for seven days; immunosuppression was discontinued thereafter. Animals immunized with the immunogenic peptides rejected their allografts in an accelerated fashion; animals immunized with the nonimmunogenic peptides or adjuvant did not develop accelerated allograft rejection. No difference existed in the time course of the rejection process when recipients were challenged with third-party BN cardiac allografts, so we deduced that the effect observed was a specific response to donor class-II MHC peptide priming. Additional studies showed that priming with the immunogenic RT1.D^β (HLA-DR-like) peptides, and specifically with the most immunogenic single peptide (RT1.D^β, residues 20-44), was responsible for the observed acceleration of the rejection process [48].

Morphologic evaluation of cardiac allografts harvested from animals at the time of rejection revealed interstitial cellular rejection of significantly greater intensity in the accelerated compared with the control groups. Interest-

ingly, in addition to the severe interstitial cellular rejection, animals primed with the immunogenic class-II MHC peptides had severe vascular rejection compared with control animals. Specifically, allografts harvested from immunogenic class-II MHC allopeptide-primed recipients showed classic morphologic features of vascular rejection with necrotizing arteritis, including fragmentation of the elastica, fibrinoid necrosis, and a mild perivascular mixed cellular infiltrate. Allografts from this group showed deposition of IgG (predominantly IgG2b), C3, and fibrin throughout the vasculature. By contrast, acute rejection in unprimed rats was cellular in nature, with normal vessels on histologic examination, and essentially no endothelial deposition of IgG, C3, or fibrin. Our morphologic and immunohistologic data suggest an important role for alloantibodies in the accelerated vascular rejection observed in animals primed with immunogenic donor class-II MHC allopeptides.

We also examined the T-cell proliferative response to the peptides in primed and control animals. Interestingly, we observed lymphocyte proliferation against peptides known to be immunogenic in both the peptide-immunized animals and in the animals primed *in vivo* by the transplanted organ, as previously described [32, 33]. However, the degree of proliferation to the peptides was significantly higher in the peptide-immunized group. Thus, peptide immunization markedly increased the precursor frequency of allopeptide-reactive T-cells compared with that seen in *in-vivo* priming as a result of rejecting a graft [48]. This increased precursor frequency might play an essential role in initiating the effector mechanisms that accelerate the rejection process (Fig. 3).

To study DTH mechanisms, we generated alloreactive T-cell clones by *in-vivo* priming via the indirect pathway [49]. Inbred LEW rats were primed *in vivo* by immunization in the foot pad with the immunogenic synthetic class-II MHC allopeptide (RT1.D^β, residues 20-44). This is the same peptide that alone is effective in priming animals to accelerate allograft rejection [48]. One week later, we harvested the primed lymphocytes from the draining lymph nodes, and established an RT1.D^ββ20-44 T-cell line by repeated stimulation with RT1.D^ββ20-44 peptide presented by responder APCs. The T-cell line proliferated significantly to the RT1.D^ββ20-44 peptide but not to a specificity control peptide derived from the RT1.B locus (RT1.B^ββ20-44) when presented by self APCs. In addition, the T-cell line produced significant amounts of IFN- γ but not IL-4 upon restimulation with the RT1.D^ββ20-44 peptide. By limiting dilution, we then generated T-cell clones from this Th1 line. Flow cytometric analysis with specific monoclonal antibodies showed that all RT1.D^ββ20-44-specific T-cell clones were CD4⁺. Six clones proliferated specifically to the RT1.D^ββ20-44 peptide and produced IFN- γ but not IL-4 when restimulated with RT1.D^ββ20-44 peptide *in vitro* (that is, Th1 clones).

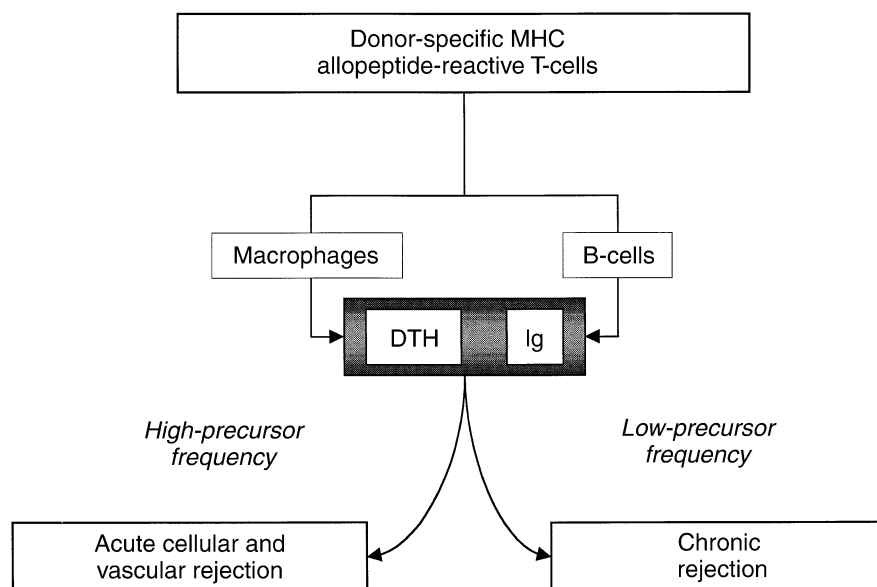


Fig. 3. Contribution of indirect allorecognition to graft rejection: A working hypothesis. (Adapted from [48] with permission.)

Using RT-PCR transcript analysis with specific rat TCR V β primers, we showed that all these clones expressed V β 9 TCR transcripts. These clones are self-restricted and do not proliferate to intact donor (WF) cells *in vitro*; thus, they recognize RT1^u alloantigens through the indirect pathway only. We then injected naïve LEW animals with 25 to 30 \times 10⁶ cells intraperitoneally of one of the RT1.D^u β 20-44-specific T-cell clones (clone 2F4). Five days later, the animals were challenged with RT1.D^u β 20-44 in the ear to check for DTH responses. These animals mounted significant DTH responses to the allopeptide, but not to control antigen. More interestingly, LEW animals injected with the 2F4 clone had significant DTH response to re-challenge with irradiated allogeneic WF spleen cells, but not to syngeneic LEW or third-party BN splenocytes. This was the first demonstration that MHC allopeptide-specific Th1 cell clones transfer a DTH response. The specific response to WF cells indicates processing and presentation of allo-MHC by self APCs *in vivo*.

We recently focused our studies on generating T-cell clones from animals primed *in vivo* by rejection of vascularized allografts, because this is the physiologic pathway of priming to donor-derived peptides [50]. We generated self-restricted class-II MHC allopeptide-specific T-cell clones (to the same peptide, RT1.D^u β 20-44) from the spleen and kidney of LEW (RT1^l) rats undergoing acute rejection of MHC-incompatible WF (RT1^u) renal allografts. All the clones that specifically proliferated to the peptide (RT1.D^u β 20-44) were CD4⁺ and produced IFN- γ but not IL-4 upon restimulation with the peptide *in vitro*. The Th1 clones from splenic and renal T-cell lines of rejecting animals expressed a restricted TCR V β

repertoire: V β 4, 8.2, or 9. In comparison, the clones generated from T-cell lines of RT1.D^u β 20-44-immunized LEW rats all expressed TCR V β 9 only. Adoptive transfer of T-cell clones expressing TCR V β 9 or V β 8.2 to naïve LEW animals elicited significant DTH responses after challenge with the RT1.D^u β 20-44 peptide or allogeneic WF (RT1^u) splenocytes. By contrast, TCR V β 4-expressing clones elicited no DTH response. These data indicate that not all Th1 clones are pathogenetic; some clones transfer DTH responses while others do not. This important observation might explain why priming animals with the RT1.B peptides, although immunogenic, did not accelerate allograft rejection [48]. The exact reasons why a clone is not pathogenetic are unknown, but preliminary studies suggest that pathogenicity is related to affinity of the particular TCR binding to the peptide+MHC complex on APCs [50].

In summary, CD4⁺ T-cells primed by donor peptides via the indirect pathway help monocytes and B-cells to effect DTH responses and produce donor-specific allo-antibodies, respectively. These cellular and humoral mechanisms contribute to graft rejection. It is also possible that such CD4⁺ T-cells help activate CD8⁺ cytotoxic T-cells which, through direct allorecognition of class-I MHC-bearing donor cells, contribute to allograft destruction by cytotoxicity [40].

Human studies

The first report of self-restricted T-cell recognition of processed allo-MHC in humans was published by de Koster et al, who produced T-cell clones primed by a synthetic peptide derived from the hypervariable domain of the β chain of HLA-DR3 (residues 67–85) [51]. These

clones were capable of proliferating to the allo-MHC peptide presented by self HLA-DP class-II molecules. Liu et al used synthetic MHC peptides (20-25mer) derived from the hypervariable domain of DR β 1*0101 to establish *in vitro* a T-cell line from an HLA-DR11/DR12 responder that recognized a specific MHC allopeptide (residues 21–42) in the context of self class-II MHC [52]. This line also was capable of recognizing cells expressing DR β 1*0101, from which the peptide sequences were derived in the presence of autologous APCs, or in the absence of autologous APCs only when expressed together with DR11. These data thus suggest the self-restricted recognition of processed allo-MHC. Responder cells were shown to be CD4+ and were inhibited by specific anti-DR11 monoclonal antibody. Restricted TCR V β usage by the responding cell line also was noted [53].

Lessons learned from these *in-vitro* studies and *in-vivo* animal studies utilizing synthetic MHC peptides have been extended into the human transplant arena. Liu and colleagues have demonstrated donor-specific MHC allopeptide T-cell reactivity in humans with recurring episodes of acute cardiac allograft rejection [54]. More interesting was the demonstration of shifting T-cell responses to different allopeptides with time. Such a change in the pattern of T-cell responses has been termed epitope switching or spreading and can occur to peptides representing alternative regions within a given MHC β chain hypervariable region (intramolecular spreading) or alternatively, to peptides representing different MHC chains (intermolecular spreading) [54]. An important observation in these human studies is that indirectly primed T-cells are present at a much lower precursor frequency than are directly primed T-cells. In fact, Liu et al calculated that such allopeptide-specific T-cells are present at 100 to 1000 times lower frequency than that of cells recognizing intact (direct allorecognition) allo-MHC [24]. This finding is consistent with the hypothesis that small numbers of peptide-primed T-cells are mediating an indolent immune response that reflects the natural history of chronic rejection, a phenomenon characterized by slowly progressive organ dysfunction.

We then studied indirect allorecognition in human renal allograft recipients with chronic rejection [55]. We found that peripheral blood lymphocytes from 82% of patients who were mismatched for at least one of 3 DR molecules and who had chronic allograft dysfunction specifically proliferated to the mismatched allopeptides (N = 9/11). Proliferation was seen in only 6% of control subjects (2/33, $P < 0.0001$) (Table 1). The precursor frequency of peptide-specific T-cells was more than ten-fold higher in patients with chronic rejection as compared with controls. These data demonstrated for the first time that T-cells of patients with chronic graft dysfunction are primed to recognize and respond to specific donor-derived MHC allopeptides [55]. Our study also

Table 1. Indirect alloreactivity to donor HLA-DR peptides in renal transplant recipients with chronic allograft dysfunction^a

Group	No.	HLA-DR mismatch	Serum creatinine	Positive reactivity	
				No.	Percentage
1	11	+	3.02 \pm 0.61	9	82 ^b
2	10	+	1.79 \pm 0.15	0	0
3	5	–	2.88 \pm 0.53	0	0
4	18	–	1.48 \pm 0.11	2	11

^a Adapted from Ref. 48

^b $P < 0.0001$

demonstrated epitope spreading, results comparable to the observations of Liu et al in patients with acute cardiac allograft rejection [54]. More recent work from the same group examined the relationship among allopeptide reactivity, epitope spreading, and chronic rejection in human cardiac allograft recipients. Utilizing synthetic peptides corresponding to the hypervariable region of 32 HLA-DR alleles, they followed donor-specific MHC allopeptide lymphocyte responses in a population of 34 heart allograft recipients. T-cells from sequential samples of blood collected from the patients as long as 36 months after transplantation were studied in limiting dilution analysis for allopeptide reactivity. The incidence of coronary artery vasculopathy was significantly higher in patients who displayed persistent alloreactivity late after transplantation (after six months) than in patients who showed no alloreactivity after the first six months after transplantation. Epitope spreading was observed with an increased frequency in patients developing vasculopathy in less than two years, compared with patients without vasculopathy [56]. These studies and our own observations in renal transplant recipients [55] indicate that indirect allorecognition correlates with, and might play a key role in, chronic rejection.

SUMMARY

Taken together, these experimental observations in animals as well as humans have led to the formulation of a hypothesis linking MHC-allopeptide-primed T-cells and chronic rejection (Fig. 4). Small numbers of indirectly primed T-cells are present and targeted against a restricted repertoire of immunodominant peptides in the immediate post-transplant period. Concomitant with the possible decline in the importance of directly primed T-cells with time post engraftment [40, 57], the precursor frequency of indirectly primed T-cells continues to be low grade. In addition, naïve CD4+ T-cells recognize new epitopes, by a yet unclear mechanisms, and are continuously becoming activated while immunosuppression is being reduced. Activated CD4+ T-cells provide help and in turn activate the effector mechanism of allograft destruction, namely, monocytes/macrophages (DTH),

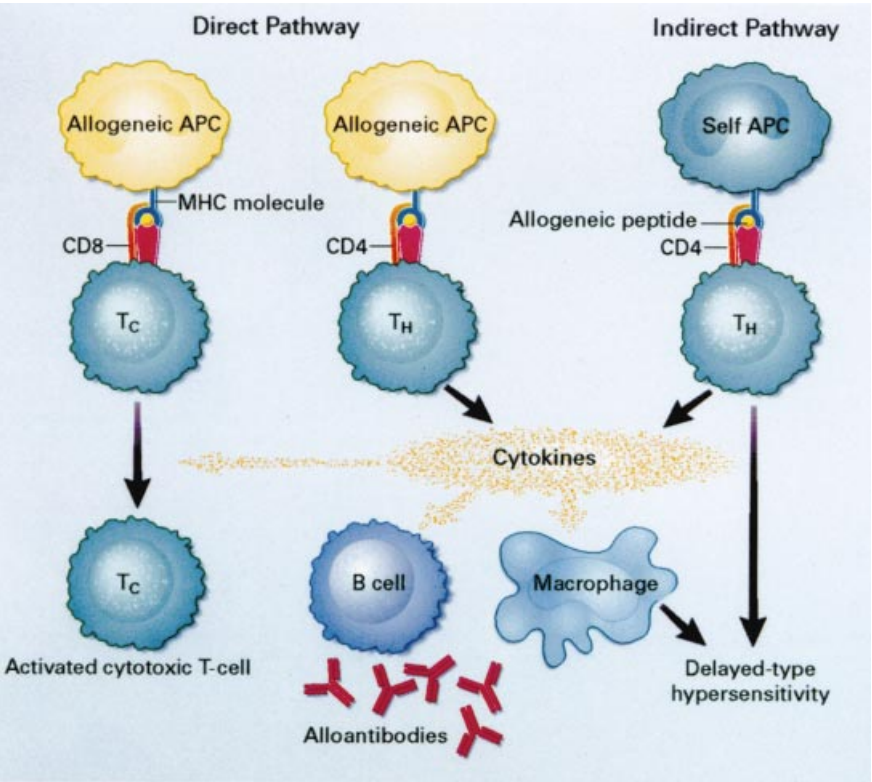


Fig. 2. Mechanisms of allorecognition and graft rejection. (Adapted from [19] with permission.)

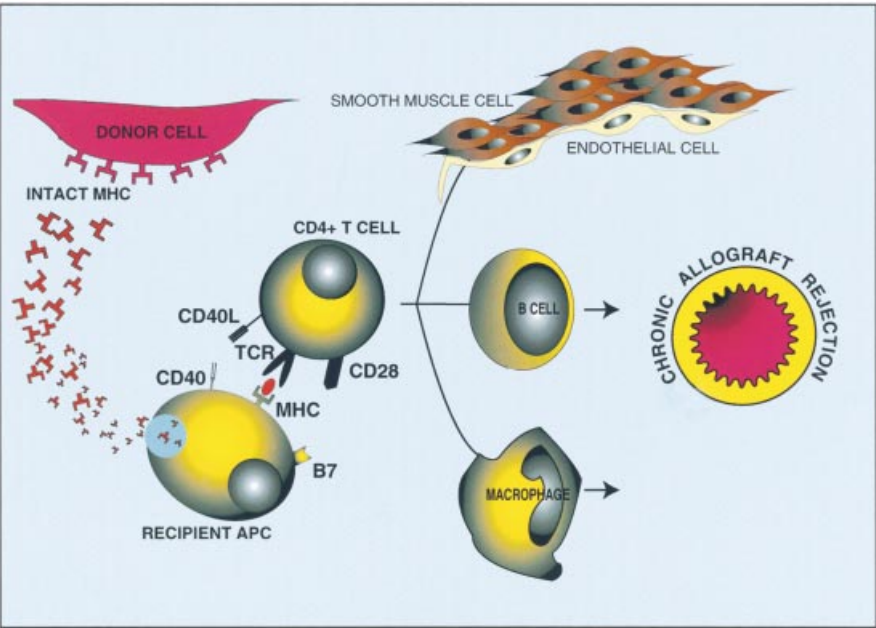


Fig. 4. Role of indirect allorecognition in chronic allograft rejection: A working hypothesis. (Adapted from [67] with permission.)

B-cells (alloantibodies), and endothelial and smooth muscle cells. Through complex cellular and molecular mechanisms [58], which include tissue injury, healing, and repair, the grafts develop morphologic changes of chronic rejection that lead to clinical organ dysfunction and failure.

We can draw several implications from this working hypothesis. First, to prevent chronic allograft rejection, we need to specifically target the indirect pathway. In addition, it is likely that such interventions will have to be introduced at a relatively early stage prior to epitope

shifting. Several strategies hold promise in that regard. In particular, strategies targeted at blocking CD28-B7 and/or CD40L-CD40 T-cell co-stimulatory activation have been shown to prevent development [59–64] and even interrupt progression [65] of chronic rejection in experimental animals. Interestingly, targeting T-cell co-stimulatory activation also might be effective in ameliorating injury mediated by alloantigen-independent mechanisms of graft dysfunction, such as ischemia/reperfusion injury [11, 12]. Obviously, the applicability of these rodent studies needs to be confirmed in large animals [66] before clinical trials in humans are begun.

QUESTIONS AND ANSWERS

DR. NICOLAOS E. MADIAS (*Chief, Division of Nephrology, New England Medical Center, Boston, Massachusetts*): Thank you for this wonderful presentation. Can you expand on the determinants of the immunogenicity of processed allo-MHC antigens? What might be the mechanism of the epitope spreading you described as a function of time?

DR. SAYEGH: I could hypothesize on potential mechanisms. One possibility, for example, is that acute rejection, infections (such as CMV), or potentially ischemic injury to the graft can upregulate MHC expression. This upregulation can lead to increased shedding of MHC molecules, which in turn can result in a different type pattern of peptide reactivity because hidden epitopes are now exposed. In addition, changes in immunosuppression or other factors with time can affect T-cell recognition of different epitopes. The bottom line is that the precise mechanisms of epitope spreading remain unknown.

DR. MADIAS: It would appear that both the distribution and the timing of expression of the various costimulatory signals are of importance. What do we know about the mechanism of expression of B7 molecules on activated APCs and endothelium and of CTLA4 inhibitory receptors on activated T-cells?

DR. SAYEGH: We know, for example, that cytokines are important in controlling the upregulation of costimulatory molecules. We recently published two studies in which we looked at ischemic injury and its effect on B7 expression [11, 12]. Both studies were in a setting in which there was no alloimmune response. We found that renal ischemic injury not only upregulates MHC expression but also upregulates B7 expression. When we gave animals with ischemic injury to their kidneys systemic CTLA4Ig, we ameliorated the ischemic reperfusion injury in the absence of an alloimmune response. We think this decrease in reperfusion injury is because of inhibition of the cytokine/chemokine surge. Not a lot is known about what regulates CTLA4 upregulation. This is a very important area because if we can figure a way to use a physiologic pathway to terminate the im-

mune response for therapeutic purposes, it may have significant clinical relevance to several immune-mediated diseases.

DR. MADIAS: Since CTLA4 provides an inhibitory signal to activated T-cells, what about a strategy of blocking the B7-CD28 pathway but allowing the engagement of B7-CTLA4 or even enhancing the expression of CTLA4 inhibitory receptor on activated T-cells?

DR. SAYEGH: This is a very important issue as well. If you target B7, then you are actually inhibiting both pathways, CD28 and CTLA4. In certain circumstances, delaying the administration of CTLA4Ig is more effective than giving it early on. Our hypothesis is that this delay allows CTLA4 to be expressed on T-cells. We have recent data showing that if we block CTLA4, we can abrogate the beneficial effects of CTLA4Ig [68]. It is possible that the timing of expression of CTLA4 vis-a-vis B7 blockade is a critical issue.

DR. ANDREW S. LEVEY (*Division of Nephrology, New England Medical Center*): It seems to me that you have shown very nicely the indirect pathway of allorecognition in various models. However, I'm not sure that you clearly differentiated its relative importance in acute versus chronic rejection, although your presentation suggests that you favor the hypothesis that it might be a dominant and unsuppressed mechanism in chronic rejection. Would you agree that the clinical data that you presented prove that the indirect mechanism of allorecognition occurs but doesn't discriminate whether this is important in acute versus chronic rejection?

On a broader scope, it seems to me that acute and chronic rejection clearly differ with regard to pathologic patterns and the immune effector pathways, but is there any evidence that the initiation of the injury differs? Is it possible that both direct and indirect allorecognition occur in all phases of rejection? Does the separation of acute and chronic rejection as clinical entities make any difference?

DR. SAYEGH: Yes, I do believe there is a difference. There's a fundamental pathophysiologic difference that we don't understand very well and that is at least manifested by the different morphology and pathology that we see. Let me go back to the issue of the relative contribution of each pathway. I do believe that both pathways are operative in the acute setting. In fact, as I said before, available data support strongly the contribution of the indirect pathway to acute rejection. But I favor the hypothesis that in the acute setting, because of the high number of antigen-presenting cells in donor organs like dendritic cells, which express high-density MHC and co-stimulatory molecules, the direct pathway is dominant. We know at the precursor frequency levels that T-cells responding via the direct allorecognition pathway are much higher in number than those responding via the indirect pathway. In the chronic phase, the importance

of the direct pathway is reduced. For example, we know that the graft lacks donor APCs several weeks after transplantation. The endothelial cell can function as a direct APC, but some data from heart transplant patients showed that many patients with chronic rejection are "tolerant" to the direct response, as suggested by the precursor frequency analysis against donor cells [57]. Because these patients cannot mount a direct response, the chronic rejection that occurs must be driven by indirect allorecognition.

At this moment, we cannot definitively answer the question of what is the relative contribution of direct versus indirect alloresponses to acute versus chronic rejection. My fundamental point is that a pathway exists that has been forgotten for years—indirect allorecognition—and that it probably plays a major role in chronic rejection. To exaggerate a bit for effect, I don't think anybody cares substantially anymore about acute rejection; the major problem now is chronic rejection.

DR. MADIAS: Is there cross-talk between the two pathways of allorecognition? Is it possible for recipient APCs to provide costimulation of T-cells that have been primed by the direct allorecognition pathway?

DR. SAYEGH: Yes, this is called trans-costimulation. If the T-cell gets a costimulatory signal from the same APC, it's called "costimulation in cis." The two pathways do talk to each other. The best demonstration of this is at the endothelial cell level. The endothelial cell is of donor origin, while the monocytes and T-cells are of recipient origin; they go through the endothelium to reach the graft. Indeed, recent data in collaboration with the laboratory of Dr. Briscoe at Children's Hospital in Boston show that endothelial cells can promote indirect allorecognition [69].

DR. JOHN T. HARRINGTON (*Dean, Tufts University School of Medicine, Boston, Massachusetts*): I have two questions. Could you give me your best estimate of the quantitative degree of importance of the indirect allorecognition pathway in chronic rejection? Is it 50/50, 90% direct, 90% indirect, or is there no way of knowing? Second, are there clinical methods that would allow us to serially measure the degree of inhibition of the indirect allorecognition pathway?

DR. SAYEGH: To answer your first question, these studies have not been done. However, we hypothesize that indirect allorecognition is the predominant pathway in chronic rejection. Whether the direct response also contributes remains unknown.

We have more data now that have not been published yet. In work similar to our rat studies, we generated T-cell lines and clones from patients. We took these cell lines from stable patients and those with chronic rejection. We found that in patients with chronic rejection, the T-cell lines secrete TH1 cytokines (IL-2, IFN- γ). The stable patients, however, secrete TH2 cytokines

(IL-4, IL-10). We are trying to simplify this method and generate what we call "mini" T-cell lines to develop new immuno-surveillance assays of transplant patients. The answer to your second question is yes, there are ways to serially measure the degree of inhibition of the indirect pathway, but they are not very well developed.

DR. ANDREW J. KING (*Division of Nephrology, New England Medical Center*): One strategy that has been employed over the years, and especially prior to the introduction of cyclosporine, was pre-transplant donor blood transfusion. There is evidence of a beneficial effect of this approach in preventing solid organ rejection, particularly with one-haplotype-matched transfusion heart transplant recipients. Could you speculate on how this might work in relation to your model? Could this practice in some way inhibit epitope shifting?

DR. SAYEGH: It has been known for years that if you give donor antigen in the form of bone marrow or blood transfusion, you can induce tolerance in the recipient animal. The limited data in humans have not been reproducible. The implication of our studies is that if one does not induce tolerance to the indirect response, prolonged survival is likely, but the patient will probably end up developing chronic rejection. This also means that any tolerance strategy that succeeds in preventing acute and chronic rejection has to induce tolerance to both direct and indirect responses. In recent studies, we showed that administration of donor antigen was necessary to induce tolerance and prevent chronic rejection [70].

DR. KING: In your review of chronic rejection, you referred to the characteristic vascular lesion with intimal proliferation and an inflammatory response. What is the nature of the cells in that lesion? Are these T-cells with CD4+ markers? Are there CD8+ cells there?

Also, you implied that antibody production and B-cells play a role in this chronic rejection process. Is there any evidence that antibody-mediated responses are occurring within the histologic lesion itself?

DR. SAYEGH: The intimal lesions themselves reflect predominantly smooth muscle cell proliferation. The smooth muscle cells around the intima proliferate and expand and they push the intima inside. Immunohistologic staining reveals the presence of CD4+ T-cells and monocytes/macrophages but not a lot of CD8+ cells. Dr. David Adams and Dr. Mary Russell have looked at the transition in cell expression in the lesion. Early on, you find mostly CD4+ T-cells, some monocytes, no or very few CD8+ T-cells; with progression of the lesion, there are usually more monocytes/macrophages, and fewer CD4+ T-cells. The IgG antibodies play a very important role; in fact, studies using the more refined ELISA assay show that you can correlate anti-donor IgG responses with the appearance of chronic rejection [71]. We think that this antibody response is a surrogate for CD4+ indirect alloresponses, as previously shown [43].

DR. ANNAMARIA KAUSZ (*Division of Nephrology, New England Medical Center*): Evidently, some data suggest that the half-life of HLA-identical kidney transplants was better in 1974 than in 1996. Could this be due to the effect of cyclosporine nephrotoxicity?

DR. SAYEGH: The number of these patients was very small, and thus a firm conclusion cannot be drawn.

DR. KAUSZ: You mentioned brain death as a possible cause of antigen-independent allograft dysfunction. Is there an immunologic mechanism behind that?

DR. SAYEGH: We recently published a paper on this issue [72]. We used a rat model of so-called "explosive" brain death. These animals developed shock and hypotension, hypoperfusion of the organs, and severe ischemic injury. The organs of these animals showed upregulation of inflammatory cytokines and chemokines within hours after the injury.

DR. BERTRAND L. JABER (*Division of Nephrology, New England Medical Center*): If one assumes that alloantigen cell-surface shedding is a prerequisite for indirect allorecognition, what are the factors that determine cell-surface shedding besides acute rejection?

DR. SAYEGH: This has not been studied.

DR. JABER: It has been shown that many of the membrane-bound cytokine receptors are cleaved off the cell surface to become soluble following activation of metalloproteinases. It would be interesting to examine whether cell-surface shedding of alloantigens is indeed regulated by putative metalloproteinases. The possible modulation of this phenomenon by metalloproteinase inhibitors could lead to the development of new therapeutic strategies that limit indirect allorecognition.

DR. SAYEGH: Yes. In support of that hypothesis, we know through association studies that levels of soluble donor MHC molecules correlate with chronic rejection.

DR. KING: One strategy employed in patients who have been labeled as having chronic rejection has been the transition from cyclosporine to other immunosuppressive agents such as FK506 or mycophenolate. Do you think that that maneuver is a useful strategy? If so, does the efficacy relate to increased immunosuppression?

DR. SAYEGH: It is certainly more immunosuppression. There is no proof that this strategy works, however. The most interesting study is one that came from the University of Maryland, where they showed that if you switch patients with renal transplants and chronic rejection from azathioprine to mycophenolate, renal function improves (abstract, Weir et al, *Transplantation* 67:S82, 1999). What we do not know is whether this improvement occurred because there was an effect on the progression of chronic rejection or because of reduction in the cyclosporine dose.

DR. MADIAS: Does administration of CTLA4Ig affect peripheral T-cell or B-cell populations or in-vitro T-cell responsiveness?

DR. SAYEGH: It binds to B7 molecules and causes T-cell anergy *in vitro*. There are no data regarding a direct effect on B-cells.

DR. HARRINGTON: When you were talking about the immunogenicity of some of the peptides, you mentioned that a difference in only two amino acids led to a different degree of immunogenicity. Do we know anything about the structure of those particular proteins and how that minimal quantitative amino acid difference leads to such a powerful functional difference?

DR. SAYEGH: We have gone beyond that and split the peptides into two peptides. Each one has only one amino acid difference, and you get the same immune response with one amino acid difference between donor and recipient [50]. That's not unusual because these are probably linear structures that are being recognized and bound to HLA molecules of the recipient APCs. What is being recognized as foreign is the substituted amino acid. The T-cell receptor thus needs only to encounter one polymorphic amino acid to mount an immune response. This phenomenon has been shown in mutation studies. You can substitute only one amino acid in a molecule and yet induce a vigorous immune response. This is not inconsistent at all.

DR. MADIAS: Could you please comment on the most promising strategies for induction and maintenance of tolerance and the potential mechanisms involved?

DR. SAYEGH: There are several strategies to try to induce tolerance, but some of the clinically promising ones include blocking T-cell costimulation, donor bone marrow chimerism, donor-specific transfusions coupled with immunomodulatory strategies, and some humanized monoclonal antibodies [73]. The translation of these strategies from small animals in the laboratory to primates and humans remains an elusive goal.

ACKNOWLEDGMENTS

The Principal Discussant is a recipient of the National Kidney Foundation Clinician Scientist Award and an American Society of Transplantation Young Scientist Award.

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REFERENCES

1. DEMETRIS AJ: Pathology of chronic rejection. *Graft* 1(Suppl 2):6, 1998
2. CARPENTER CB: Long-term failure of renal transplants: Adding insult to injury. *Kidney Int* 48:S40-S44, 1995
3. PAUL L: Chronic renal transplant loss. *Kidney Int* 47:1491-1499, 1995
4. MATAS AJ: Chronic allograft dysfunction: Clinical definitions and risk factors. *Graft* 1(Suppl 2):48-51, 1998
5. MACKENZIE HS, TULLIUS SG, HEEMAN UW, AZUMA H, RENNKE HG, BRENNER BM, TILNEY NL: Nephron supply is a major determinant of long term renal allograft survival in rats. *J Clin Invest* 94:2148-2152, 1994
6. TULLIUS SG, TILNEY NL: Both alloantigen-dependent and -independent

- dent factors influence chronic allograft rejection. *Transplantation* 59:313–318, 1995
7. CECKA JM: *The UNOS Scientific Renal Transplant Registry—Ten Years of Kidney Transplants*. Los Angeles, UCLA, 1997, pp 1–14
 8. REINSMOEN NL, JACKSON A, MCSHERRY C, WIESNER RH, KONDO M, KROM RA, HERTZ MI, BOLMAN RM, MATAS AJ: Organ-specific patterns of donor antigen-specific hyporeactivity and peripheral allogeneic microchimerism in lung, kidney, and liver transplant recipients. *Transplantation* 60:1546–1554, 1995
 9. TULLIUS SG, HANCOCK WW, HEEMAN U, AZUMA H, TILNEY NL: Reversibility of chronic renal allograft rejection: Critical effect of time after transplantation suggests both host immune dependent and independent phases of progressive injury. *Transplantation* 58:93–99, 1994
 10. SAYEGH MH, CARPENTER CB: Tolerance and chronic rejection. *Kidney Int* 51(Suppl 58):S11–S14, 1997
 11. TAKADA M, CHANDRAKER A, NADEAU KC, SAYEGH MH, TILNEY NL: The role of B7 costimulatory pathway in experimental cold ischemia/reperfusion injury. *J Clin Invest* 100:1199–1203, 1997
 12. CHANDRAKER A, TAKADA M, NADEAU KC, PEACH R, TILNEY NL, SAYEGH MH: CD28-b7 blockade in organ dysfunction secondary to cold ischemia/reperfusion injury. *Kidney Int* 52:1678–1684, 1997
 13. KRIEGER NR, DENGPIG Y, FATHMAN CG: CD4+ but not CD8+ cells are essential for allorecognition. *J Exp Med* 184:2013–2018, 1996
 14. SAYEGH MH, WATSCHINGER B, CARPENTER CB: Mechanisms of T cell recognition of alloantigen: The role of peptides. *Transplantation* 57:1295–1302, 1994
 15. SHOSKES DA, WOOD KJ: Indirect presentation of MHC antigens in transplantation. *Immunol Today* 15:32–38, 1994
 16. SAYEGH MH, CARPENTER MH: Role of indirect allorecognition in allograft rejection. *Int Rev Immunol* 13:221–229, 1996
 17. CHITILIAN HV, AUCHINCLOSS H: The indirect pathway in graft rejection. *Curr Opin Organ Transplant* 2:3–7, 1997
 18. BENICHO G, TAM RC, SOARES LRB, FEDOSEYEVA EV: Indirect T-cell allorecognition: Perspectives for peptide-based therapy in transplantation. *Immunol Today* 18:67–71, 1997
 19. SAYEGH MH, TURKA LA: The role of T cell costimulatory activation in transplant rejection. *N Engl J Med* 338:1813–1821, 1998
 20. MAGEE CC, SAYEGH MH: Peptide-mediated immunosuppression. *Curr Opin Immunol* 9:669–675, 1997
 21. CHICZ RM, URBAN RG, LANE WS, GORGA JC, STERN LJ, VIGNALI AA, STROMINGER JL: Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* 358:764–768, 1992
 22. CHICZ R, URBAN R, GORGA J, VIGNALI A, LANE W, STROMINGER J: Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* 178:27–47, 1993
 23. HUNT DF, MICHEL H, DICKINSON TA, SHABANOWITZ J, COX AL, SAKAGUCHI K, APPELLA E, GREY HM, SETTE A: Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* 256:1817–1820, 1992
 24. LIU Z, SUN Y, XI Y, MAFFEI A, REED E, HARRIS P, SUCIU-FOCA N: Contribution of direct and indirect recognition pathways to T cell alloreactivity. *J Exp Med* 177:1643–1650, 1993
 25. BRAUN YM, MCCORMACK A, WEBB G, BATCHELOR RJ: Mediation of acute but not chronic rejection of MHC-incompatible rat kidney grafts by alloreactive CD4 T cells activated by the direct pathway of sensitization. *Transplantation* 55:117–182, 1993
 26. MULUK SC, CLERICI M, VIA CS, WEIR MR, KIMMEL PL, SHEARER GM: Correlation of *in vitro* CD4+ T helper cell function with clinical graft status in immunosuppressed kidney transplant recipients. *Transplantation* 52:284–291, 1991
 27. SCHULIK RD, WEIR MR, MILLER MW, COHEN DJ, BERMAS BL, SHEARER GM: Longitudinal study *in vitro* CD4+ T helper cell function in recently transplanted renal allograft patients undergoing tapering of their immunosuppressive drugs. *Transplantation* 56:590–596, 1993
 28. SCHULIK RD, MULUK SC, CLERICI M, BERMAS BL, VIA CS, WEIR MR, SHEARER GM: Value of *in vitro* CD4+ T helper cell function test for predicting long-term loss of human renal allografts. *Transplantation* 57:480–482, 1993
 29. SHERWOOD RA, BRENT L, RAYFIELD LS: Presentation of alloantigens by host cells. *Eur J Immunol* 16:569–574, 1986
 30. DALCHAU R, FANGMANN J, FABRE JW: Allorecognition of isolated, denatured chains of class I and class II major histocompatibility complex molecules: Evidence for an important role for indirect allorecognition in transplantation. *Eur J Immunol* 22:669–677, 1992
 31. SAYEGH MH, KHOURY SK, HANCOCK WW, WEINER HL, CARPENTER CB: Induction of immunity and oral tolerance with polymorphic class II MHC allopeptides in the rat. *Proc Natl Acad Sci USA* 89:7762–7766, 1992
 32. WATSCHINGER B, GALLON L, CARPENTER CB, SAYEGH MH: Mechanisms of allorecognition: Recognition by *in vivo* primed T-cells of specific major histocompatibility complex polymorphisms presented as peptides by responder antigen-presenting cells. *Transplantation* 57:572–577, 1994
 33. GALLON L, WATSCHINGER B, MURPHY B, AKALIN E, SAYEGH MH, CARPENTER CB: Indirect pathway of allorecognition: The occurrence of self-restricted T cell recognition of allo-MHC peptides early in acute allograft rejection and its inhibition by conventional immunosuppression. *Transplantation* 59:612–616, 1995
 34. BENICHO G, TAKIZAWA PA, OLSON CA, McMILLAN M, SERCARZ EE: Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J Exp Med* 175:305–308, 1992
 35. BENICHO G, FEDOSEYEVA E, OLSON CA, GREYSEN HM, McMILLAN M, SERCARZ EE: Limited T cell response to donor MHC peptides and the prevention of allograft rejection. *J Immunol* 153:938–946, 1994
 36. FANGMANN J, DALCHAU R, SAWYER GJ, PRIESTLY CA, FABRE JW: T cell recognition of donor major histocompatibility complex class I peptides during allograft rejection. *Eur J Immunol* 22:1525–1530, 1992
 37. BENHAM A, FABRE JW: Fine specificity of peptide determinants for indirect T cell recognition of class I MHC alloantigens. *Transplantation* 58:1236–1240, 1994
 38. SHIRWAN H, LEAMER M, WANG HK, MAKOWKA L, CRAMER DV: Peptides derived from α -helices of allogeneic class I major histocompatibility complex antigens are potent inducers of CD4+ and CD8+ T cell and B cell responses after cardiac allograft rejection. *Transplantation* 59:401–410, 1995
 39. GHOBBIAL R, HAMASHIMA T, KLOC M, ETKIN L, STANISLAW S, KAHAN B: Membrane bound or soluble truncated RT1.Aa rat class I major histocompatibility antigens induce specific alloimmunity. *Transplantation* 60:602–610, 1995
 40. VELLA JP, VOS L, CARPENTER CB, SAYEGH MH: The role of indirect allorecognition in experimental late acute rejection. *Transplantation* 64:1823–1824, 1997
 41. AUCHINCLOSS HJ, LEE R, SHEA S, MARKOWITZ JS, GRUSBY MJ, GLIMCHER LH: The role of “indirect” recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Natl Acad Sci USA* 90:3373–3377, 1993
 42. LEE RS, GRUSBY MJ, GLIMCHER LH, WINN HJ, AUCHINCLOSS HJ: Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes *in vivo*. *J Exp Med* 179:865–872, 1994
 43. STEELE DJR, LAUFER TM, SMILEY ST, ANDO Y, GRUSBY MJ, GLIMCHER LH, AUCHINCLOSS HJ: Two levels of help for B cells alloantibody production. *J Exp Med* 183:699–703, 1996
 44. DALLOUL AH, CHMOUZIS E, NGO K, FUNG-LEUNG W-P: Adoptively transferred CD4+ lymphocytes from CD8-/- mice are sufficient to mediate rejection of MHC class II or class I disparate skin grafts. *J Immunol* 156:4114–4119, 1996
 45. FANGMANN J, DALCHAU R, FABRE JW: Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J Exp Med* 175:1521–1529, 1992
 46. BENHAM AM, SAWYER GJ, FABRE JW: Indirect T cell allorecognition of donor antigens contributes to the rejection of vascularized kidney allografts. *Transplantation* 59:1028–1032, 1995
 47. KRENSKY A, WEISS A, CRABTREE G, DAVIS M, PARHAM P: T-lymphocyte–antigen interactions in transplant rejection. *N Engl J Med* 322:510–517, 1990
 48. VELLA JP, MAGEE C, VOS L, RENNKE H, HANCOCK WW, CARPENTER CB, SAYEGH MH: Indirect allorecognition of donor class II MHC

- peptides induces vascular rejection of cardiac allografts. *Transplantation* 67:1523–1532, 1999
49. CHEN W, MURPHY B, WAAGA AM, WILLETT TA, RUSSELL ME, KHOURY SJ, SAYEGH MH: Mechanisms of indirect allorecognition in graft rejection: class II MHC allopeptide specific T cell clones transfer delayed type hypersensitivity responses *in vivo*. *Transplantation* 62:705–710, 1996
 50. WAAGA A, CHANDRAKER A, SPADOFORA-FERREIRA M, IYENGAR A, KHOURY SJ, CARPENTER CB, SAYEGH MH: Mechanisms of indirect allorecognition: Characterization of MHC class II allopeptide-specific T helper clones from animals undergoing acute allograft rejection. *Transplantation* 65:876–883, 1998
 51. DE KOSTER HS, ANDERSON DE, TERMITJELEN A: T cells sensitized to synthetic HLA-DR3 peptide give evidence of continuous presentation of denatured HLA-DR3 molecules. *J Exp Med* 169:1191–1196, 1989
 52. LIU Z, BRAUNSTEIN NS, SUCIU-FOCA N: T cell recognition of allopeptides in context of syngeneic MHC. *J Immunol* 148:35–40, 1992
 53. LIU Z, SUN YK, XI YP, HONG B, HARRIS EP, REED FE, SUCIU-FOCA N: Limited usage of T cell receptor V beta genes by allopeptide-specific T cells. *J Immunol* 150:3180–3186, 1993
 54. LIU Z, COLOVAI AI, TUGULOA S, REED EF, FISHER PE, MANCINI D, ROSE EA, CORTESINI R, MICHLER RE, SUCIU-FOCA N: Indirect recognition of donor HLA peptides in organ allograft rejection. *J Clin Invest* 98:1150–1157, 1996
 55. VELLA JP, FERREIRA M, MURPHY B, ALEXANDER SI, HARMON W, CARPENTER CB, SAYEGH MH: Indirect allorecognition of MHC allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 64:795–800, 1997
 56. CIUBOTARIU R, LIU Z, COLOVAI AI, HO E, ITESCU S, RAVALLI S, HARDY MA, CORTESINI R, ROSE EA, SUCIU FN: Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest* 101:398–405, 1998
 57. HORNICK PI, MASON PD, YACOB MH, ROSE ML, BATCHELOR R, LECHLER RI: Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. *Circulation* 97:1257–1263, 1998
 58. RUSSELL ME, RAISANEN-SOKOLOWSKI A, KOGLIN J, GLYSING-JENSEN T: Immunobiology of chronic rejection: Effector pathways that regulate vascular thickening. *Graft* 1(Suppl 2):7–10, 1998
 59. AZUMA H, CHANDRAKER A, NADEAU K, HANCOCK WW, CARPENTER CB, TILNEY NL, SAYEGH MH: Blockade of T cell costimulation prevents development of experimental chronic allograft rejection. *Proc Natl Acad Sci USA* 93:12439–12444, 1996
 60. RUSSELL ME, HANCOCK WW, AKALIN E, WALLACE AF, GLYSING-JENSEN T, WILLETT T, SAYEGH MH: Chronic cardiac rejection in the Lewis to F344 rat model: Blockade of CD28-B7 costimulation by CTLA4Ig modulates T cell and macrophage activation and attenuates arteriosclerosis. *J Clin Invest* 97:833–838, 1996
 61. CHANDRAKER A, RUSSELL ME, GLYSING-JENSEN T, WILLETT TA, SAYEGH MH: T cell costimulatory blockade in experimental chronic cardiac allograft rejection: Effects of cyclosporine and donor antigen. *Transplantation* 63:1053–1058, 1997
 62. GLYSING-JENSEN T, RAISANEN-SOKOLOWSKI A, SAYEGH MH, RUSSELL ME: Chronic blockade of CD28-B7 mediated T cell costimulation by CTLA4Ig reduces intimal thickening in MHC class I and II incompatible mouse heart allografts. *Transplantation* 164:1641–1645, 1997
 63. SAYEGH M, ZHENG X-G, MAGEE C, HANCOCK W, TURKA L: Donor antigen is necessary for the prevention of chronic rejection in CTLA4Ig-treated murine cardiac allografts. *Transplantation* 64:1646–1650, 1997
 64. HANCOCK WW, BUELOW R, SAYEGH MH, TURKA LA: Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat Med* 4:1392–1396, 1998
 65. CHANDRAKER A, AZUMA H, NADEAU K, CARPENTER CB, TILNEY NL, HANCOCK WW, SAYEGH MH: Late blockade of T cell costimulation interrupts progression of experimental chronic allograft rejection. *J Clin Invest* 101:2308–2318, 1998
 66. KIRK AD, HARLAN DM, ARMSTRONG NN, DAVIS TA, DONG V, GRAY GS, HONG X, THOMAS D, FECHNER JH, KNECHTLE SJ: CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci USA* 94:8789–8794, 1997
 67. VELLA JP, KNOFLACH A, WAAGA AM, SAYEGH MH: T cell mediated immune responses in chronic rejection: Role of indirect allorecognition and costimulation. *Graft* 1(Suppl 2):11–17, 1998
 68. JUDGE TA, WU Z, ZHENG X-G, SHARPE AH, SAYEGH MH, TURKA LA: The role of CD80, CD86 and CTLA-4 in alloimmune responses and the induction of long-term allograft survival. *J Immunol* 162:1947–1951, 1999
 69. DENTON MD, GEEHAN CS, ALEXANDER SI, SAYEGH MH, BRISCOE DM: Endothelial cells modify the costimulatory capacity of transmigrating leukocytes and promote CD28-mediated CD4⁺ T cell alloactivation. *J Exp Med* 190:555–566, 1999
 70. SAYEGH MH, ZHENG X-G, MAGEE C, HANCOCK WW, TURKA LA: Donor antigen is necessary for the prevention of chronic rejection in CTLA4Ig-treated murine cardiac allograft recipients. *Transplantation* 64:1646–1650, 1997
 71. KERMAN RH: Can we predict chronic rejection? Humoral immune monitoring assays. *Graft* 1(Suppl 2):66–68, 1998
 72. TAKADA M, NADEAU K, HANCOCK WW, MACKENZIE HS, SHAW GD, WAAGA AM, CHANDRAKER A, SAYEGH MH, TILNEY NL: Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation* 65:1533–1542, 1998
 73. DENTON MD, MAGEE CC, SAYEGH MH: Immunosuppressive strategies in transplantation. *Lancet* 353:1083–1091, 1999